Original Research Article

Dark Purple Luem Pua rice extract reduces disease severity in rat models of inflammatory bowel disease

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Abstract

Purpose: To investigate the effects of an aqueous extract of unpolished dark purple glutinous Thai rice, variety Luem pua (LP), in two rat models of inflammatory bowel disease (IBD).
Methods: Polyphenolic compounds content were determined by HPLC methods and antioxidant activities by DPPH and FRAP assays of the LP extract. The effect of the LP extract at 5 g/kg/day were evaluated in two rat models of IBD that included acetic acid and indomethacin induced IBD. On each day of treatment, changes of body weight, stool consistency and stool blood were scored and expressed as disease activity index (DAI). At the end of the experiments, the animals were euthanized. Colon length and spleen weight were determined, and the degree of inflammation of the colon was scored.
Results: Rats in both models of IBD (acetic acid- and indomethacin-induced IBD), exhibited significant increases in DAI, macroscopic inflammation scores and spleen weights, while the lengths of colon were decreased. Pretreatment with LP extract attenuated the disease severity in both models as seen by the reverse of all observed altered parameters.
Conclusion: These data suggest that LP extract might be beneficial in preventing and/or treating IBD.

Keywords: Inflammatory bowel disease (IBD), Dark purple glutinous rice Var. Luem Pua, Acetic acid, Indomethacin

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of intestinal disorders which entail chronic inflammation of digestive tract especially in many parts of small bowel and/or colon. Clinical features of IBD include diarrhea, passage of blood and mucus in stool, abdominal pain, fever and weight loss. Complications of IBD are closely associated with an increased risk of colorectal cancer [1]. Two main subtypes of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Although the etiology and pathogenesis of IBD are not fully understood, many evidence suggest that IBD is associated with genetics, immune dysregulation, intestinal barrier function destruction and gut microbiota alteration.

In preclinical studies, many chemicals are used to induce IBD models in animals, among them, acetic acid and indomethacin induced IBD models are the most widely used [2] and are suggested to mimic UC and CD [3]. Generally, the goal of IBD treatment is to reduce the inflammation that triggers signs and symptoms of the disease and usually involves either drug therapy or surgery. At present various groups of drugs are used to treat IBD, including anti-inflammatory drugs, immune modulators, and antibiotics. These drug therapies, however, may have many adverse effects associated with long-term use [4]. Therefore, increased research efforts have been devoted to the treatment or prevention of IBD using natural or alternative treatments.

Rice (*Oryza sativa*) is a principal food for over half of the world's population, especially in Asia. Many varieties of pigmented rice have been developed in many countries including Thailand and are recognized for their nourishing phytochemical content [5]. Dark purple glutinous rice, variety *Luem Pua* (LP), is a special variety of glutinous rice with dark-purple pericarp and originally grown by the hill tribe called Hmong. Luem Pua rice extract was shown to have high antioxidant activity, enriched with polyphenolic compounds [6] and memory enhancing effects [7]. Recently, evidence have been reported on oxidative stress as one of the important roles in the pathogenesis and progression of IBD. It has been reported that reactive oxygen species (ROS) upregulate the expression of genes involved in innate and adaptive immune responses in the GI tract and also induce gastroduodenal ulcers, IBD, and gastric and colorectal cancers [8]. This study aimed to investigate the effects of LP extract on chemically induced inflammatory bowel diseases in rats.

EXPERIMENTAL

Plant extract

Dark purple glutinous rice, variety Luem Pua, was obtained from Phitsanulok Rice Research Center, Wangthong, Phitsanulok, Thailand. Luem Pua (LP) rice was soaked with hot water (60-70 ºC) (10 g/100 ml) for 5 min and the extract was filtered through multiple layers of gauze and cotton. The extraction procedure was repeated 5 times and the filtrate was collected and lyophilized with freeze dryer. The percent yield of the LP extract was 2.16%. The dry powdered extract was stored in a sealed dark bottle and kept at 4 ºC and freshly dissolved in distilled water before being used. Doses used in this study were expressed as weight of dried rice seed.

Polyphenolic compound determination

The polyphenolic compounds in the LP extract were measured using reversed-phase high performance liquid chromatography (RP-HPLC) based on the method previously described [9]. The standards were dissolved in HPLC grade ethanol at different concentrations and passed through a 0.22 µm sterile filter before the injection. The flow rate was set at 0.8 mL/min and injection volume was 20 µL. The mobile phase was a binary solvent system consisting of methanol and 1 % acetic acid/water. The detector was set at 280 nm mainly for phenolic acids and at 360 nm for flavonoids.

DPPH radical scavenging (DPPH) assay

The scavenger activity of LP extract was investigated using stable radical 2,2 dihenyl-1-picrylhydrazyl (DPPH) assay as previous described [10, 11] with some modifications. In brief, the reaction mixtures (200 µL), consisted of 100 µL of 0.1 mM solution of DPPH in ethanol and 100 µL of various concentrations of LP extract (25, 50, 100, 200 and 400 µg/mL) or L-ascorbic acid reference standards (1, 2.5, 5, 10 and 25 µg/mL) that were plated in 96-well microtiter plates. After incubation for 20 min at room temperature the changes in color from deep violet to light yellow were measured at 517 nm using a spectrophotometer (Elisa Plate Reader sunrise, TECAN). The reduction absorbance was plotted against the concentration of the sample and the IC_{50} values were determined using Sigma Stat 3.5 software. Inhibition of DPPH was calculated according to Eq 1.

Inhibition (%) = $(A_0 - A_1 /A_0)100$ ………… (1)

where A_0 is the absorbance of the control (DPPH alone) and A_1 is the absorbance of reactions containing LP extract.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay based on the reducing power of a compound was used in this study to measure the total antioxidant power of LP extract as described earlier with some modifications [12]. In brief, the FRAP reagent was freshly prepared by adding 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-Tris (2-pyridyl)-1,3,5-triazine) dissolved in 40 mM of HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1 (v/v). L-Ascorbic acid at 1, 2.5, 5,

10 and 25 µg/mL was used as the antioxidant standard. The reaction mixture (200 µL) consisting of 100 µL of FRAP reagent and 100 µl of various concentrations of LP extract (25, 50, 100, 200 and 400 µg/mL) were plated in 96-well microtiter plate, mixed well and then absorbance was measured at 620 nm. The antioxidant capacity was based on the ability to reduce ferric ions of the extract and was expressed as µmol of ascorbic acid equivalent (AAE/g extract).

Experimental animals

Male Wistar rats (6 weeks old, 250 - 300 g body weight) were purchased from Nomura Siam International Co. Ltd, Pathumwan, Bangkok, Thailand. The animals were housed in stainless steel cages and maintained in an air-conditioned room (25 ± 1 °C) with a 12 h light: 12 h dark cycle and fed a standard chow diet (Chareon Pokapan Co. Ltd. Thailand) with drinking water *ad libitum*. All animals were acclimatized to the laboratory room and handling for 1 week before the start of the experiments. All procedures complied with the standards for the care and use of experimental animals and approved by the Animal Ethics Research Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand (Recorded no. ACUC-KKU-21/2560).

Acetic acid-induced IBD in rat model

The rats were divided into 3 groups of 6 rats each. Animals were oral gavage infused with distilled water or LP extract (5 g/kg) for 7 consecutive days. On day 6 of treatment, 4 % acetic acid was administered transrectally as previous described [13]. The rats were anesthetized with xylazine (5 mg/kg each, i.p.) and were rectally administered 2 mL of 4% acetic acid solution using a soft polypropylene tube which was advanced 6 cm from the anus. The rats were in the Trendelburg position during this process, and kept for 1-2 min in head down position to prevent leakage. After then, acetic acid was flushed with 5 mL of 0.9 % normal saline. On each day, the body weights and DAI of each rat were recorded. After 48 h of acetic acid administration, rats were euthanized by deep ether anesthesia. The colon lengths and spleen weights were measured. Then, colon tissues were mounted on a plain paper and the inflammation was recorded as macroscopic ulcer score [14].

Indomethacin-induced IBD in rat model

The rats were divided into 3 groups of 6 rats each. Animals were oral gavage infused with either distilled water as the control or LP extract (5 g/kg) once daily for the experimental period of 11 days. On day 8 and day 9 of treatment, animals were injected subcutaneously with distilled water or 7.5 mg/kg indomethacin [15]. On each day, the DAI of each animal was recorded.

At the end of treatment period, all animals were euthanized by deep ether anesthesia and the colons and spleens were removed. The colon lengths and spleen weights were measured. Macroscopic inflammation scores were assigned based on clinical features of the ilea and colons using a scale of 0 - 8 according to the method previously described [16].

Determination of disease activity index (DAI)

Three parameters, body weight loss, stool consistency and the presence of blood in stool were observed each day and scored as described earlier [17]. Summation of the scores of all 3 parameters were expressed as DAI.

Statistical analysis

All the results are presented as mean ± SEM. Statistical comparison between groups was analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Content of phenolic compounds in LP extract

The HPLC study showed that the LP extract contained many biologically active phenolic compounds. The content of the compounds presented in the extract were cyanidin-3-glucoside (C3G), peonidin-3-glucoside (P3G), ferulic acid, catechin, isoquercetin, rutin, quercetin, gallic acid and tannic acid in the amounts as shown in Table 1.

DPPH antioxidant activity

LP extract inhibited DPPH activity in a dose-dependent manner Table 2). The percentage of inhibition of DPPH activity by LP extract ranged from 5.05 ± 1.30 to 95.87 ± 0.14 % at an LP extract between 25 and 400 µg extract/mL final concentrations. In this study, the IC_{50} values of ascorbic acid and LP extract were 9.97 and 164.92 µg/mL.

Table 1: Content of biological active phenolic compounds in LP extract

Phenolic compound	mg/kg of LP extract
1. Anthocyanins	
- *Cyanidin-3-glucoside* (C3G)	4,840.68
- *Peonidin-3-glucoside* (P3G)	507.98
2. *Ferulic acid*	177.32
3. *Catechin*	27.49
4. *Isoquercetin*	14.42
5. *Rutin*	11.67
6. *Quercetin*	8.33
7. *Gallic acid*	8.11
8. *Tannic acid*	7.17

Table 2: DPPH scavenging activities of LP extract

LP extract concentration (µg/mL)	Inhibition (%, mean ± SEM.)
25	5.05 ± 1.30
50	20.43 ± 3.16
100	47.36 ± 0.26
200	69.84 ± 0.62
400	95.87 ± 0.14

FRAP antioxidant activity

The antioxidant capacity of LP extract determined by FRAP assay is shown in Table 3. The ascorbic acid equivalent (AAE) of the extract was calculated to be 178.25 µmol AAE/g extract.

Effect of LP extract on acetic acid -induced IBD in rats

Colonic transrectal administration of acetic acid (represented UC) triggered intense inflammatory responses in the large bowel, characterized by high DAI and macroscopic ulcer score when compared to the control group. Treatment with LP extract at 5 g/kg/day significantly decreased, although not completely antagonized, the effect of acetic acid on DAI (Figure 1A) and macroscopic ulcer score (Figure 1B) when compared to the acetic acid-treated group.
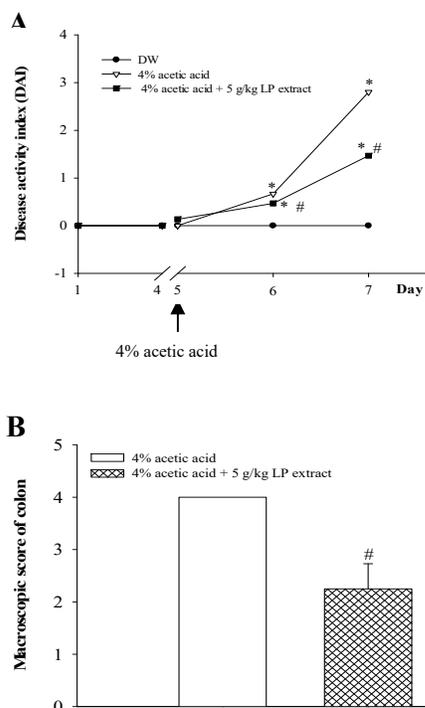

Figure 1: Effect of LP extract on DAI (A) and macroscopic score of colons (B) in acetic acid-induced IBD in rats. Data are expressed as mean ± SEM; * significantly different when compared to the control group; # significantly different when compared to the acetic acid treatment group

The rats treated with acetic acid showed a significant decrease in colon lengths when compared to the control group (Figure 2). Pretreatment with LP extract (5 g/kg/day) significantly reduced the effect of acetic acid on colon lengths.

In this study, acetic acid-induced IBD rats showed spleen enlargements and a significant increase in the spleen weights (Figure 3). Interestingly, rats treated with 5 g/kg/day LP extract for 5 days, significant reduction of spleen weights were observed when compared to acetic acid treatment group.
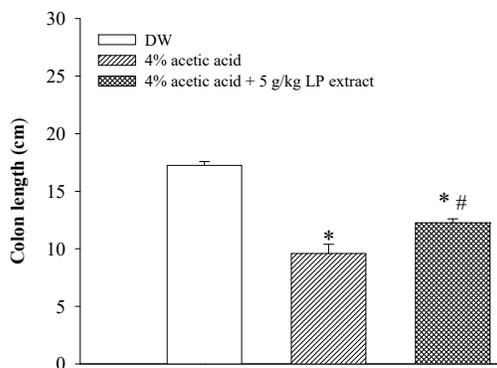
Table 3: The antioxidant capacities of LP extract determined by FRAP assay

Ascorbic acid		LP extract		Antioxidant capacity of LP (µmol AAE/g extract)
Concentration (µg/mL)	*Absorbance (620 nm)*	*Concentration (µg/mL)*	*Absorbance (620 nm)*	
1	0.073	25	0.019	
2.5	0.166	50	0.048	
5	0.345	100	0.069	178.25
10	0.544	200	0.122	
25	1.030	400	0.254	

Figure 2: Effect of LP extract on colon lengths in acetic acid-induced IBD in rats. Data are expressed as mean ± SEM; *significantly different when compared to the control group. # significantly different when compared to acetic acid treatment group
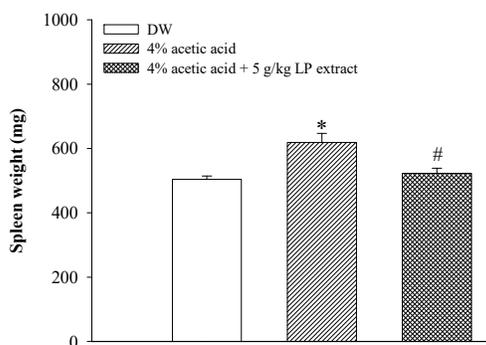

Figure 3: Effect of LP extract on spleen weight in acetic acid-induced IBD in rats. Data are expressed as mean ± SEM. *Significantly different when compared to the control group. # significantly different when compared to acetic acid-treated group

Effect of LP extract in indomethacin-induced IBD in rats

Within 48 h of indomethacin administration to rats (representing CD), inflammation of the intestines and colons could be seen and significant increases in the DAI and macroscopic score were observed. Interestingly, pretreatment with LP at 5 g/kg/day before indomethacin administration showed a significant reduction in DAI (Figure 4A) and the macroscopic inflammation score (Figure 4B) that suggested improvement in the intestinal lesions.

The colon lengths of indomethacin-treated rats was significantly shorter than those of the control group. Treatment with 5 g/kg/day LP extract reduced the effects of indomethacin on colon lengths with a significant increase in the colon lengths compared to lengths of the control (Figure 5).
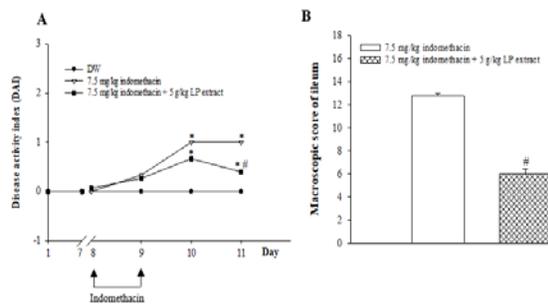

Figure 4: Effect of LP extract on DAI (A) and macroscopic score of ilea (B) in indomethacin-induced IBD in rats. Data are expressed as mean ± SEM. * Significantly different when compared to the control group. # Significant differently when compared to the indomethacin group
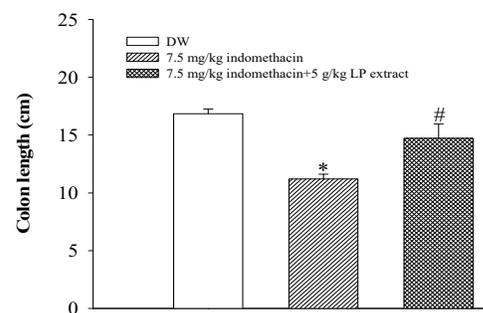

Figure 5: Effect of LP extract on colon lengths in indomethacin-induced IBD in rats. Data are expressed as mean ± SEM. *Significantly different when compared to the control group. # Significantly different when compared to the indomethacin group

The indomethacin-induced IBD rats had a significant increase of spleen weights when compared to the control group. Treatment with LP 5 g/kg/day produced a significant decrease in spleen weights when compared to indomethacin-treated group (Figure 6).
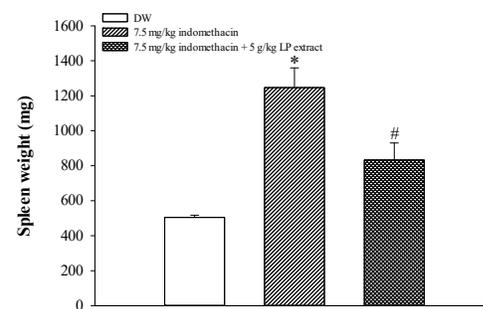

Figure 6: Effect of LP extract on spleen weights in indomethacin-induced IBD in rats. Data are expressed as mean ± SEM. * Significantly different when compared to the control group. #Significantly different when compared to the indomethacin group

DISCUSSION

The present study showed that the aqueous extract of LP contained many biologically active polyphenolic compounds and possessed significant antioxidant activity in DPPH and FRAP assays. Pretreatment with LP extract significantly ameliorated the severity of inflammation in acetic acid- and indomethacin-induced IBD in Wistar rats.

Among the two models, acetic acid and indomethacin, of induced IBD models, different mechanism of actions for progression and pathogenesis of the diseases occur. Acetic acid creates chemical injury to the mucosal epithelium that induces a transient phenotype mimicking UC while indomethacin model is more likely to mimic CD [3]. Interestingly, LP extract showed a significant decrease of DAI, the shortening of colon length, the enlargement of spleens and the inflammations of ilea and/or colons suggest that LP extract could reduce the severity and progression of IBD.

Evidence is increasingly shown that oxidative stress, increased ROS generation and decreased antioxidant activities, have roles in the pathogenesis and progression of IBD. Several reports have shown the upregulation of ROS and reactive nitrogen species (RNS) in IBD patients as well as in animal models [8]. Hence, antioxidant treatment has been suggested as additional treatment in IBD patients [18]. Many plant flavonoids are shown to suppress the inflammation in both *in vivo* and *in vitro* studies, thus, reducing the severity of different inflammatory diseases including IBD [19, 20]. Containing many biologically active flavonoids, including anthocyanins, ferulic acid, catechin, isoquercetin, rutin, quercetin, gallic acid and tannic acid, LP extract might help reduce the severity of IBD by acting through the effects of antioxidant, anti-inflammation and immunomodulation.

Many previous studies in animal models of IBD showed that the impairment of parasympathetic nerve functions might have effects on acute inflammation associated with the immune dysregulation by the release of higher levels of pro-inflammatory cytokines [21]. Activation of muscarinic cholinergic receptors on intestinal mucosal epithelial cells could attenuate cytokine activities in an animal model of colitis [22] and decrease inflammatory cytokines-induced epithelia barrier dysfunction [23]. An *in vitro* study of the present group of authors [24], using isolated guinea pig ileum, suggested that LP extract caused ileum contraction in a dose-dependent manner and was completely inhibited by atropine, an antagonist of the muscarinic receptor of acetylcholine. To the best of these present authors knowledge, no plant polyphenolic compound is suggested to act at muscarinic cholinergic receptor. It might be the case that LP extract contains certain substance that have ability to directly activate muscarinic cholinergic receptors to reduce pro-inflammatory cytokine release and help reduce the severity of inflammation in IBD.

Moreover, the alteration of the compositions and functions of the gut microbiota have roles in the pathogenesis of IBD [25]. The gut microbiota have physiological functions associated with immune system and maintenance of intestinal homeostasis. A recent study showed that LP rice had a high survival rate of probiotic bacteria compared with other Thai rice cultivars [26] and black rice also had prebiotic activity in regulating the intestinal environment [27]. Thus, the current authors are now evaluating the effects of LP extract on the changing of microbiota in guts of IBD in animal models.

CONCLUSION

The results of this study demonstrate that LP extract possesses antioxidant activity and decreases the severity of inflammation in both acetic acid- and indomethacin-induced IBD in rats. Thus, LP extract is a potential candidate for the development as a therapeutic agent for the prevention and/or treatment of IBD.

DECLARATIONS

Acknowledgement

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors contributed to the conceptualizations of this

manuscript. Kornsuda Thipart performed the experiments, collected and analyzed the data and wrote the first draft of the manuscript, Acharaporn Na Lampang Noenplab prepared rice sample, Kutcharin Phunikhom analyzed the data and Jintana Sattayasai had primary responsibility for supervision, project administration and critically revised the manuscript. All authors have read and approved the manuscript for publication.

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